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Thank you.

MINH TAM DAVIS
ART UNIT 1642, ROOM 8A01, MB 8E12
305-2008

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Quantitative Investigation of Cardiomyocyte Hypertrophy and Myocardial Fibrosis Over 6 Years After Cardiac Transplantation

ARTHUR T. ARMSTRONG, PHD,* PHILIP F. BINKLEY, MD, FACC,* PETER B. BAKER, MD,†
P. DAVID MYEROWITZ, MD, FACC,‡ CARL V. LEIER, MD, FACC*

Columbus, Ohio

Objectives. This study was performed to determine the degree and time course over 6 years of cardiomyocyte hypertrophy and myocardial fibrosis of the cardiac allograft in transplanted patients.

Background. Diastolic dysfunction and to a certain extent systolic dysfunction are common cardiac findings after heart transplantation. The development of posttransplant cardiomyocyte hypertrophy and myocardial fibrosis likely contributes to these derangements.

Methods. Cardiomyocyte diameter and percent fibrosis were determined in serial endomyocardial biopsy specimens obtained from 1 month up to 6 years following heart transplantation in 50 patients. Endomyocardial biopsy specimens from 40 patients with primary dilated cardiomyopathy and 11 normal subjects were similarly analyzed for control data. Analyses were performed in a blinded format using a validated computerized image analysis system (Optimas 5.2).

Results. Early (1 month) cardiomyocyte enlargement decreased to the smallest diameter 6 months posttransplant, but thereafter progressively increased by 10% to 20% over the subsequent 5- to 6-year period. Although not statistically established, principal stimuli may include a discrepancy in body size (recipient > donor), coronary allograft vasculopathy and posttransplant systemic hypertension. Percent myocardial fibrosis rose early (1 to 2 months) posttransplant and thereafter remained at the same modest level of severity.

Conclusions. Cardiomyocyte diameter of the transplanted heart gradually increases over time, while percent myocardial fibrosis rises early and remains in a modestly elevated plateau after 2 months posttransplant. These histostructural changes likely contribute to the hemodynamic and cardiac functional alterations commonly observed posttransplant.

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It is generally accepted that cardiac allografts undergo some cardiomyocyte hypertrophy and myocardial fibrosis after transplantation (1–3). While the specific causes for these changes have not been definitively established, it is likely that the histologic alterations contribute to the functional changes of the transplanted heart, particularly myocardial stiffness, diastolic dysfunction and, to a certain extent, systolic dysfunction (4–11).

The time course and extent of cardiomyocyte hypertrophy and interstitial fibrosis in human transplanted hearts have not been determined. We hypothesized that these histostructural changes increase in a progressive manner throughout the posttransplant course. This blinded retrospective study employed computerized morphometric methodology to analyze serial endomyocardial biopsies obtained over 6 years from 50 patients with cardiac allografts for the purpose of testing this hypothesis. Biopsies from 40 patients with dilated cardiomyopathy and 11 subjects with normal cardiac function were analyzed under the same conditions to provide control data for comparison.

Methods

Study populations. The posttransplant study group represents all patients who survived 5 years or more after orthotopic cardiac transplantation at The Ohio State University Medical Center; these 50 posttransplant patients consist of 35 men and 15 women with a mean (± 1 SD) age of 45 ± 10 years (range 12 to 59 years). The pretransplantation cardiac diagnoses were atherosclerotic heart disease for 22 patients, nonischemic dilated cardiomyopathy for 26 patients and hypertrophic cardiomyopathy for 2 patients. All underwent cardiac transplantation at The Ohio State University Medical Center from 1986 to 1991 such that serial endomyocardial biopsy specimens were available for posttransplant periods ≥5 years.

The cardiac allograft biopsies used for this study were taken from the 50 recipients at set intervals following transplantation; specifically at 1, 2, 4, 6, 9 and 12 months, and every 6 months out to 5 years posttransplant for all 50 patients and out to 6 years for 30 of them.

Two control groups provided endomyocardial biopsy samples for comparison. Eleven subjects, 7 men and 4 women with

From the *Division of Cardiology, †Department of Pathology and ‡Division of Cardiac Surgery, The Ohio State University College of Medicine, Columbus, Ohio. The Harold Zieg Memorial Research Fund supported this investigation.

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Address for correspondence: Dr. Carl V. Leier, Division of Cardiology, The Ohio State University Hospitals, 669 Means Hall, 1654 Upham Drive, Columbus, Ohio 43210.

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a mean age of 33 ± 10 (range 17 to 50) years, with no demonstrable clinical or anatomic (echocardiography) heart disease served as the normal control group. These subjects underwent heart biopsy as part of an oncologic, prechemotherapy protocol. Forty patients, 21 men and 19 women with a mean age of 40 ± 14 (range 18 to 68) years, afflicted with primary dilated cardiomyopathy of a duration ranging from 1 month to 8 years served as the dilated cardiomyopathy control group. All patients of this group underwent diagnostic cardiac catheterization and echocardiography. Patients with historical, clinical or laboratory evidence of systemic hypertension, valvular disease, occlusive coronary artery disease or other nonmyocardial structural disorders were excluded.

After written informed consent was obtained for each procedure, four right ventricular-septal endomyocardial biopsy specimens were taken at each biopsy procedure via standard technique (12) using a disposable Cordis 54 cm, 2.2 mm forceps bioptome. All specimens were placed immediately (< 10 s after forceps closure) in 10% formalin and subsequently embedded in a single paraffin block. Sections were sliced 4-μm thick and mounted on glass slides. A slice taken from the specimen depth of 60% to 70% was then stained with Masson trichrome for microscopic visualization and image analysis.

Morphometric analysis. Field sampling by light microscopy was used to select the region for analysis. One microscopic field was isolated per biopsy slide as a representation of that biopsy. The fields were generally taken from the midportions of the biopsy sample, purposely avoiding areas of inflammation, vasculitis, ischemic change, ischemia-induced fibrous replacement, crush-distortion artifact and scar tissue from a prior biopsy. Each field for analysis had to contain predominately longitudinally sectioned cardiomyocytes. The sampling fields were digitized to a computer database via a light microscope (Zeiss, Thornwood, NY) through a 20× objective lens, coupled to a high resolution color camera (Hitachi model No. HV-C11, Woodbury, NY) that interfaced with an IBM-compatible Pentium computer (Everex, Freemont, CA). The digitized biopsy fields were then magnified 760× and analyzed using Optimas 5.2 image analysis software (Optimas Inc., Edmonds, WA) (see Fig. 1, panel A). The image analysis software was calibrated using a stage micrometer (Klarmann Rulings, Manchester, NH) traceable to the National Institute of Standards Technology; the mean of 14 measurements was within 0.06 μm of the stage micrometer standard.

The myocyte diameters within the field were measured using standard criteria (13). A point-to-point perpendicular line was placed across the longitudinally cut myocyte at the level of the nucleus (Fig. 1, B) and this diameter length was then measured by the computer-imaging software. All of the longitudinally directed myocytes with a distinct cell border (at the level of the nucleus) within the sampling field were measured and averaged to provide the mean cardiomyocyte diameter. Transverse or oblique cut myocytes were excluded. Cell width was determined from longitudinally positioned myocytes to reduce the error of determining such for myocytes that may not be precisely cut perpendicular to their long axis.

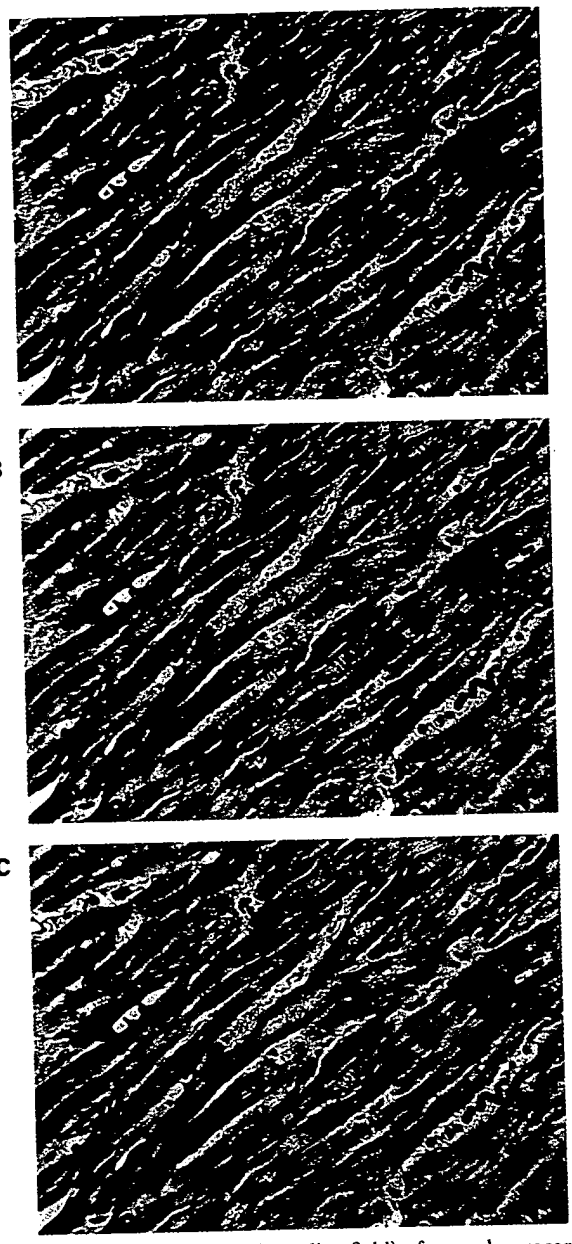

Figure 1. A, Region of interest (sampling field) of an endomyocardial biopsy specimen (Masson trichrome stain). **B, Perpendicular measurement lines** were placed across sectioned horizontal cardiomyocytes at the level of midnucleus. The line lengths were measured and averaged by computer software to provide the mean myocyte diameter of the biopsy specimen. **C,** Using the operator's threshold settings, the myocardial fibrous tissue was highlighted (**blue**) and measured by computer software as percent of myocardial tissue. The white nontissue spaces were similarly demarcated, measured and subtracted to provide a corrected area of true myocardial tissue.

The interstitial fibrosis within the same selected field of each biopsy was then quantified. The imaging software, programmed to recognize color and distinct shades, highlighted pixels of a particular color or shade we specified within the field. Fibrosis becomes blue with Masson trichrome stain,

which the imaging software highlighted within the field (based on the operator's threshold settings) and calculated the amount of area it occupied (Fig. 1, C). The white, nontissue spaces of the field were recognized (with operator-threshold settings) by the software and subtracted to provide a corrected area of total myocardial tissue in the field. The ratio of the area of fibrotic tissue to total myocardial tissue area (× 100%) was then calculated to provide a measure of percent fibrosis.

Because the tissue samples were removed by biopsy from living patients, the methodology does not lend itself to perfusion fixation, analysis of myocardium from multiple transmural or biventricular sites and other methodological considerations. Left ventricular biopsies add unacceptable risk. Despite the inability to employ perfusion fixation, control contracture or stretch of the cardiomyocytes, sample multiple sites and so forth, all biopsy specimens were obtained, processed and analyzed in the very same manner to render reasonable validity to changes in mean values. Because of methodological differences, the data of this investigation may not necessarily correspond closely to those obtained from hearts removed at necropsy or explanted during surgery (if the data from these sampling conditions should become available).

Statistical analysis. To test the reproducibility of our quantitative techniques, we chose the sampling fields from 12 randomly selected biopsies of different transplant recipients and determined interobserver and intraobserver variability. The two investigators (C.V.L. and A.T.A.) who performed the sample analyses for the entire study independently measured in blinded fashion the 12 fields for both percent fibrosis and myocyte diameter. The two investigators differed by a mean of 2.9% (representing 0.46% in absolute fibrosis) for fibrosis and 2.7% (representing 0.43 μm in absolute length) for myocyte measurements. These two investigators then blindly analyzed the same 12 fields a week or more later to compare the values with their original determinations. Investigator 1 (C.V.L.) differed by 5.4% (0.87% in absolute fibrosis) and 1.5% (0.23 μm in absolute length) from his first set of fibrosis and myocyte measurements while investigator 2 (A.T.A.) differed by 5.2% (0.75% in absolute fibrosis) and 3.3% (0.55 μm in absolute length) from his original set.

Statistical analyses of the data were performed under the guidance and consultation of the Biometrics Laboratory of the School of Public Health of The Ohio State University (Dr. Mel Moeschberger, director). One way analysis of variance (ANOVA) was used to statistically compare interstitial fibrosis and myocyte diameter among the three patient populations. Repeated measures ANOVA with posttesting was employed to determine whether numerical change in the mean values for percent fibrosis and myocyte diameter over time within the transplant group was statistically significant. Regression analyses were applied to determine whether the posttransplant data (percent fibrosis and myocyte diameter) were associated with various potential provocative factors; factors which achieved a $p < 0.10$ by univariate analysis were then tested by multivariate analysis. All data are presented as mean ± 1 SD and a statistically significant difference in mean values required a $p < 0.05$.

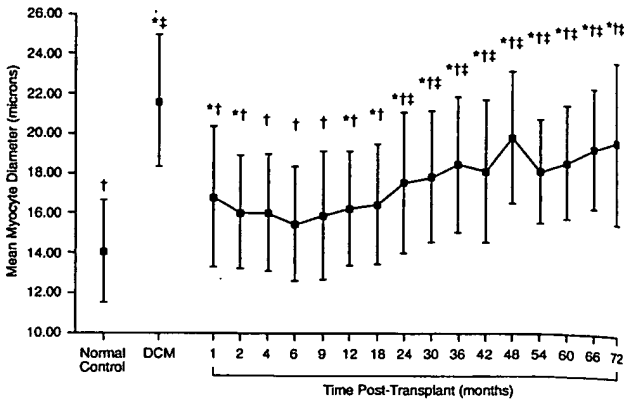

Figure 2. Mean (± 1 SD) cardiomyocyte diameters at serial time points after cardiac transplantation in 50 patients up to 60 months and 30 patients at 72 months. Data from 40 dilated cardiomyopathy (DCM) patients and 11 normal control subjects were obtained for comparison. *$p < 0.05$ vs. normal control; †$p < 0.05$ vs. DCM; ‡$p < 0.05$ vs. baseline (6 months after transplant).

Results

Myocyte diameter. The mean myocyte diameters of the myocardial biopsies analyzed at each time point posttransplant and for the normal and cardiomyopathy control groups are presented in Figure 2. Based on the assumption that early myocyte enlargement may be related to the effects of the donor's clinical condition, myocardial edema, harvest ischemia and other factors, the biopsies taken at 6 months posttransplant showed the smallest mean myocyte diameter and were therefore used as the baseline for comparison with subsequent time points. Relative to this consideration, the earliest posttransplant biopsies (1 month) had mean myocyte diameters that were numerically and almost statistically ($p = 0.0523$) larger than those of the 6-month samples. Repeated measures ANOVA indicated a statistically significant increase in mean cardiomyocyte diameter over time following the 6-month baseline nadir. For individual mean values at various time points, post-ANOVA testing showed that a statistical increase in myocyte diameter occurred at 2 years ($p < 0.01$) and remained so at all subsequent time points. After transplant, the mean myocyte diameter steadily increased as shown in Figure 2 and verified by interpoint statistical analysis (e.g., the increase from 12 to 36 months posttransplant achieved a $p < 0.0001$).

The mean myocyte diameter of the allograft baseline (6 months) was not significantly different from that of the normal control group. However, the allograft myocyte diameters at 1 year posttransplant and beyond were all statistically larger than the mean myocyte diameter of the normal controls. The mean myocyte diameter of the dilated cardiomyopathy group was significantly larger than that of the normal controls and larger

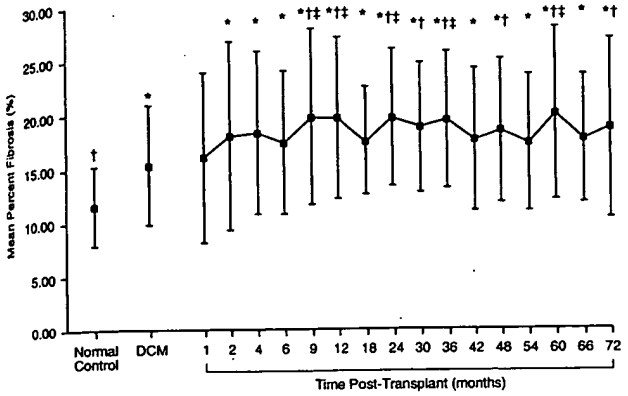

Figure 3. Mean (± 1 SD) percent fibrosis of endomyocardial biopsy specimens obtained serially after cardiac transplantation (n = 50 at 60 months and n = 30 at 72 months) and from 40 patients with primary dilated cardiomyopathy (DCM) and 11 normal control subjects. *p < 0.05 vs. normal control; †p < 0.05 vs. DCM; ‡p < 0.05 vs. baseline (1 month after transplant).

than all of the allograft mean diameters throughout the 6 year posttransplant course.

Interstitial fibrosis. The mean percent fibrosis of the biopsies taken from the posttransplant, normal control and dilated cardiomyopathy groups are presented in Figure 3. After transplant, the lowest mean percent fibrosis was noted at 1 month, and this time point was therefore used as the allograft fibrosis baseline for statistical comparisons. Although mean percent fibrosis increased numerically by 1 month (1- vs. 2-month values) and numerically remained above the baseline mean value throughout the posttransplant period (p = 0.06 by repeated measures ANOVA), only the biopsies at 9 months and 1, 2, 3 and 5 years posttransplant showed an amount of fibrosis which was statistically greater than baseline (each <0.05 with post-ANOVA testing). The mean change in percent fibrosis did not increase progressively over time after transplantation; at 2 months and beyond, the posttransplant mean values for percent fibrosis did not differ statistically from each other.

Although percent fibrosis over time tended to remain modestly elevated above the 1-month baseline for the transplanted patients, mean percent fibrosis at all time points ≥2 months posttransplant was statistically greater than that of the normal controls and at various time points ≥9 months after transplant tended to be greater than that observed for the dilated cardiomyopathy group (Fig. 3).

Potential Provocative Factors for Cardiomyocyte Hypertrophy and Myocardial Fibrosis

Donor hearts. The heart donors consisted of 47 men and 3 women with a mean age of 25 ± 8 years (range 11 to 48). The mean donor body weight was 75.7 ± 12.5 kg (range 53 to 109). There was no direct relationship between donor body weight and percent fibrosis or myocyte diameter. However, disparate donor-recipient body weights may have influenced myocyte cell diameter, although not at p < 0.05. Respective changes in myocyte diameter from baseline to 2 years for donor body weights 10 kg > recipient or mean donor/recipient weight ratios of 1.30 ± 0.20 (range 1.11 to 1.95) (n = 16), within 10 kg of recipient weight or mean donor/recipient weight ratios of 1.01 ± 0.06 (range 0.90 to 1.10) (n = 26) and for recipient weights 10 kg > donor or mean donor/recipient weight ratios of 0.80 ± 0.07 (range 0.65 to 0.89) (n = 8) were 1.41, 1.89 and 4.69 μ (p = 0.16) and from baseline to 5 years were 2.65, 2.73 and 5.42 microns (p = 0.18). No consistent changes were noted over time for mean percent fibrosis relative to donor-recipient body weights or ratios.

The ischemic time of the donor heart averaged 140 ± 54 min (range 42 to 340). Biopsies from both early (before 6 months) and later periods (after 6 months) after transplantation were analyzed relative to donor ischemic times; no correlations were found between ischemic time and the amount of myocardial fibrosis or myocyte diameter.

Medications. The transplanted patients were taking varying amounts of standard immunosuppression medication, namely cyclosporine, azathiaprine and prednisone. The respective daily mean doses of cyclosporine, azathiaprine and prednisone given at the 5-year posttransplant point were 4.24 ± 1.81 mg/kg (range 2.02 to 8.21), 122 ± 57 mg (range 25 to 225) and 10 ± 6 mg (range 5 to 20). For control of systemic hypertension over the 5- to 6-year follow-up period, 76% took a diuretic daily for a minimum of 3 years, 56% a calcium-channel blocking agent, 52% an angiotensin-converting enzyme inhibitor, 32% a beta-adrenergic blocker and/or another agent in 22%. No relationships were clearly demonstrable between immunosuppressive drug, antihypertensive agent or dose for either and myocardial fibrosis or myocyte diameter; with respect to cyclosporine, this applies to dose at each biopsy, cumulative dose and blood level.

Posttransplant clinical conditions. Other potential contributory factors for myocardial histologic change following transplantation, including systemic hypertension, cardiac allograft vasculopathy and cellular rejection, are shown in Table 1. Systemic hypertension was defined as an outpatient blood pressure recording >140/90 mm Hg. Cardiac allograft vasculopathy was declared when the annual quantitated coronary angiograms and/or intracoronary ultrasound studies showed epicardial coronary lesions with >50% diameter narrowing or distal coronary artery attenuation. Rejection was defined as grade II, grade III or grade IV at any time during the given year. Each of these factors was analyzed separately (univariate and then multivariate analysis when applicable) with respect to changes in fibrosis and myocyte diameter. No statistically significant relationships were found between the presence or absence of hypertension, coronary vasculopathy or rejection and interstitial fibrosis or cardiomyocyte diameter. However, there was a trend (p = 0.103) for an increase in myocyte diameter from baseline to 5 years posttransplant in the 11 patients with coronary vasculopathy (4.75 ± 4.34 μm) vs. those without angiographic vasculopathy (2.67 ± 3.58).

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Table 1. Occurrence of Systemic Hypertension (Systolic Pressure >140 or Diastolic >90 mm Hg), Coronary Vasculopathy, and a Rejection Grade of II, III or IV in the 50 Transplanted Patients Over Their 5 to 6 Year Posttransplant Course

Patient #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1 YR	H,C,R		H	R	H,R	R	H,R	R		R				H	H			H							
2 YR	H,C				H		H	H,R			H						H	H			H	R			H
3 YR	H,C	H			H		H	H		H	H	H		C		H	H	H			H	H		H	H
4 YR	H,C	H,C			H		H	H		H	H	H	H	C	H,R	H	H			H	H			H	
5 YR	H,C	H,C	H	H	H		H		C	H,C,R		H	C	C		H	H			H		C		H	H
6 YR	H,C	C	H		H	H,R	H	H,C	C	H,C	H	H,C	C	H,C	H	H	H	H	H	H		C			
Patient #	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50
1 YR				H				H			R	H	H	H	R	H,R		H				R			
2 YR		H	H	H	R		H	H	H		H	H	H		H			H				H		H	
3 YR		H	H	H			H	H,C				H	H,R	H	H					H		H			
4 YR		H,C	H	H	H,R	H	H	H,C	H		H			H		H								H	C
5 YR	H	H,C	H		H		H,C	C		H			H	H	H									C	C
6 YR	H	H,C	H		H	H	H,C	H,C					H	H			H								C

C = coronary vasculopathy; H = hypertension; R = rejection; YR = year.

Discussion

The hypothesis that cardiomyocyte diameter and myocardial fibrosis increase progressively throughout the posttransplant period is supported only in part by the results of this investigation. Cardiomyocyte diameter gradually increased from 6 months to 6 years after transplant. In contrast, interstitial fibrosis rose within 2 months after transplant with little additional change over the subsequent 5 to 6 years.

Myocyte diameter. The current data correspond well with those of a previous report (2) analyzing cardiomyocyte diameter at a single time point 3 years after transplantation; specifically, a mean value of 20.3 ± 3.0 μm was reported compared with 18.4 ± 3.4 μm of the current study. The authors are not aware that comparable histologic data of biopsies are available elsewhere for sequential time points posttransplant.

While determining the posttransplant provocative factors for myocyte enlargement and myocardial fibrosis was not a primary objective of this study, we found, similar to previous reports (1–3,14), little to no correlation between cardiomyocyte diameter and donor body size, immunosuppressive agent or dose, donor ischemic time or the number of rejection episodes. Albeit not statistically significant, change in myocyte diameter tended to vary inversely with the donor/recipient body weight ratio; this complements the report by Mather et al. (15) showing that undersized hearts usually enlarge to meet the needs of the recipient. In addition, the data of our investigation suggest that substantial coronary allograft vasculopathy may serve as a stimulus for posttransplant cardiomyocyte enlargement, but at a $p = 0.103$ level. Although there was no obvious statistical relationship between cardiomyocyte diameter and posttransplant blood pressure, the high prevalence of blood pressure recordings above 140/90 mm Hg posttransplant is rather striking (Table 1), a finding consistent with blood pressure patterns reported for most other posttransplant populations (5–9,11). In contrast to normal populations and the normal control group of this study, the blood pressure of posttransplant patients is infrequently <130/80 mm Hg. Elevation in systemic blood pressure, as well as the ventricular-vascular uncoupling which results in impedance mismatch between the transplanted denervated ventricle and recipient aorta-vasculature, likely serve as stimuli to cardiomyocyte enlargement. It is difficult to exclude a direct effect of immunosuppressive medications; these agents, particularly cyclosporine and corticosterioids, at least contribute indirectly by evoking a large part of the systemic hypertension noted posttransplant.

The mean cardiomyocyte diameter of the dilated cardiomyopathy group, comparable to that previously reported from this laboratory using a manual method (13), was still larger than all of the mean myocyte diameters along the entire 6-year course of the posttransplant hearts. This difference may in part be attributable to the rather high ventricular wall stress and numerous other physical, neurogenic and hormonal stimuli in the patient with dilated cardiomyopathy.

As far as the authors are aware, the reduction in cell width

over the first 6 months posttransplant is a new observation. Data are not available in this report to define a mechanism. Cellular recovery and repair and reestablishment of microvascular blood flow over the first few months posttransplant are reasonable considerations.

Interstitial fibrosis. The extent of myocardial fibrosis (15% to 19%) posttransplant in the current study is somewhat higher than the myocardial collagen fraction (4% to 10%) previously reported (2,3,14). This disparity is likely related to differences in analytical techniques in that the methodology of the current study measures total fibrosis irrespective of its components and it eliminates nonmyocardial space (e.g., sizable vasculature, clear nontissue spaces) to provide a corrected value for total myocardium. The mean percent fibrosis of the current dilated cardiomyopathy group is comparable to that of the dilated cardiomyopathy patients previously reported from this laboratory using a manual, grid point-counting technique (13).

Percent fibrosis increased within 2 months posttransplant, a finding also noted by Pickering and Boughner (3). Rather than representing a pathophysiologic process, it is possible that percent fibrosis rose simply because cardiomyocyte diameter decreased during this time period, allowing more fibrous tissue to occupy the sampling field.

After the initial rise in percent fibrosis, there was little additional increase over the subsequent 6-year posttransplant period. Another study using a mouse cardiac allograft model supports this finding by showing an increase in percent fibrosis in transplanted hearts that was immediate and did not progressively increase over time (16). This, of course, does not mean that total cardiac fibrosis does not increase with time posttransplant. In fact, no change in percent fibrosis indicates that total cardiac fibrous tissue must increase in proportion to the usual increase in cardiac weight and ventricular wall thickness. The current study does not elucidate a distinct fibrosis-inducing factor or mechanism. That proportional fibrous deposition accompanies the myocardial and cellular hypertrophic process is a reasonable view. On the other hand, the development of interstitial fibrosis may be largely independent of myocyte growth with differing responses in fibrosis type, extent and manifestations in individual patients with different predisposing factors (17).

Limitations of the study. As in all retrospective studies, the investigators were not able to rigidly control variables such as medications, doses and blood pressure, nor can the transplanted group be properly or adequately matched with other posttransplant groups to control for relevant independent variables. Patients who expired before arriving at the 5-year posttransplant study point were not included; their data may have modified the data of this report. For the normal control group, the investigators cannot guarantee that the neoplastic condition did not affect the cardiomyocyte diameter or percent fibrosis of the individual. Biopsies were taken only from the right side of the interventricular system and some of the factors studied might primarily affect the left ventricle (e.g., systemic hypertension, coronary vasculopathy); however, studies from this laboratory indicate that histopathologic changes seen in right ventricular-septal specimens parallel those noted along the left side of the septum and the left ventricular free wall and are directly influenced by left heart events and interventions (13,18). The methodology employed measured only cardiomyocyte diameter and not total cardiomyocyte area or volume. The change in width may not indicate a change in other cellular dimensions. The reader may refer to Methods for additional methodological considerations.

We conclude that there is a progressive increase in cardiomyocyte diameter in heart transplant patients up to 6 years posttransplant. A nonprogressive fibrotic response also occurs in the transplanted heart, perhaps as early as 1 to 2 months posttransplant. The structural changes likely affect short- and long-term cardiac function and possibly complicate the posttransplant course. These data provide the basis for future studies exploring the mechanisms for the observed myocyte hypertrophy and myocardial fibrosis after transplantation.

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